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Characterization of water contribution to excimer laser ablation of collagen

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Abstract

In order to gain an obvious insight into the role of water in the mechanism of the excimer laser ablation of the cornea, we have macroscopically investigated the ablation behavior of collagen gel in the swelled state by direct photoetching using an ArF excimer laser with time-resolved photography, and furthermore, the thermal effects on the microscopic structures of the collagen molecules by FTIR–ATR spectroscopy. The hydrated collagen film (HF) has a smaller threshold fluence than the dried collagen film (DF). From the time-resolved photographs, the ejected materials were detected only for HF. It was predicted that the effect of bubble formation for HF contributes to the etching. The FTIR–ATR spectroscopic results revealed that the existence of the water suppressed the denaturation of the collagen to gelatin on the surface in the irradiated region. Overall, it was inferred that during the ablation process for HF, the laser energy would be mostly consumed as the latent heat of evaporation of water, that is, the water in the gel matrix would contribute to the suppression of the increment in the temperature in the irradiated region.

Keywords: Collagen; Collagen gel; Denaturation; Excimer laser ablation

1. Introduction

Excimer laser ablation has recently attracted a great deal of attention for medical applications, in particular, corneal surgery. Photorefractive keratectomy (PRK), one of the corneal surgery methods using excimer lasers, is becoming widespread [1–4]. This method includes etching of the cornea to modify its refractive index. The cornea contains about 70% water [1]. In a previous paper, we reported that the formation of a conical structure on the dried rabbit cornea induced by excimer laser irradiation was observed by a SEM technique, whereas for the hydrated cornea, a similar surface structure could not be observed [5]. Furthermore, we predicted that the thermal denaturation of collagen → gelatin was significantly responsible for the formation of the structure. It has been expected that water in the polymer matrix is involved in the ablation mechanism of the polymers, such as biomaterials [6]. Various theoretical models, such as a thermal model and a bubble formation model, have been proposed to interpret the effect of water in biological tissues during the excimer laser ablation processes [4,7,8]. However, there is still not a clear understanding due to the structural complexity of the tissue. In addition to this issue, the microscopic structural changes have also not been explored. Hence, in order to gain an obvious insight into the role of water in the ablation mechanism, focusing on collagen as the main component of the cornea, we have macroscopically investigated the ablation behavior of collagen gel in the swelled states by direct photoetching using an ArF excimer laser with time-resolved photography. Moreover, the thermal effects on the microscopic structures of the collagen molecules at the ablation site have been scrutinized using FTIR–ATR spectroscopy.

2. Experimental

2.1. Materials

Collagen films were prepared by the cast method using type I atelocollagen (KOKEN) extracted from calf skin.

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Their film thickness was about 70 μm. Gelatin was prepared by denaturing the collagen. Gelatin films were prepared by casting a 2% aqueous gelatin at 60°C, followed by the evaporation of water.

2.2. DSC experiments

All thermal characterizations of the collagen were performed using a Seiko Instruments DSC220C differential scanning calorimeter (DSC). The temperature range chosen in this study was from 25 to 250°C. The normally used heating rate was 10°C/min.

2.3. Laser irradiation system

Laser irradiation was carried out using an excimer laser system, LEXTRA50 (Lambda Physik) operated at 193 nm (ArF). The pulse duration was 17 ns in FWHM. The laser beam was homogenized with a combination of cylindrical lenses and passed through an aperture to obtain a region of uniform intensity. The laser irradiation was carried out under atmospheric conditions for the dried films and under a relative humidity above 80% for the hydrated films. The dried and hydrated collagen films were designated DF and HF, respectively.

2.4. Atomic force microscopy (AFM) measurements

The AFM used was a Nanoscope III (Digital Instruments) and operated in the contact mode under atmospheric conditions for the DF and in water for the HF.

2.5. FTIR–ATR spectroscopy and Fourier self-deconvolution method

Infrared spectra were recorded using a Bio-Rad Digilab FTS-165 Fourier-transform infrared spectrometer and a cell with a Ge ATR prism crystal. Spectra of the films were collected at room temperature. The spectral resolution was 4 cm^{-1} and 256 scans were averaged for each spectrum.

The amide I band of collagen was Fourier self-deconvoluted by the method developed by Kauppinen et al. [9]. The parameters for deconvolution, K and 2σ, were 1.5 and 16.2 cm^{-1}, respectively. Curve-fitting calculations were performed using the Spectra Calc program (Galactic, NH). The proportions of the secondary structures were estimated from the decomposed components of the amide I band.

2.6. Nanosecond time-resolved photography

Time-resolved photography was performed with a charge-coupled-device (CCD) camera (PCO Computer Optics, SensiCam) [10]. Fluorescence of a methanol solution of Rhodamine 101 was used as the flash lamp. The second harmonic of the Nd^{3+}:YAG laser was used to excite the solution. The timing between the excimer laser, Nd^{3+}:YAG laser, and the CCD camera was synchronized via a delay generator (Stanford Research, DG535). The laser irradiation was carried out in air.

3. Results and discussion

3.1. Physical properties

The denaturation temperatures for the DF and HF were 200 and 48°C, respectively. The existence of water in the collagen matrix is suggested to reduce the denaturation temperatures corresponding to the melting of the triple helices into a random structure. The thickness of the HF was greater than five times that of the DF. The HF contained about 90 wt.% water.

3.2. AFM results

Fig. 1(a) and (b) shows the AFM images of the non-irradiated surfaces for the DF and HF, respectively. The DF has no irregularities and no obvious damage such as scratches. The value of the mean roughness (R_a) for the DF was 40 nm, whereas the R_a value for the HF increased to 120 nm due to the swelling. Fig. 1(c) shows the AFM image of the DF irradiated at a fluence of 0.10 J cm^{-2} with 1 shot. A square hole was created on the surface. The etching depth was 160 nm. A well-defined periodic structure was created in the irradiated region. This periodic structure was observed on specifically non-fused materials such as polythiophene films [10]. Fig. 1(d) shows the AFM image of the HF irradiated at a fluence of 0.10 J cm^{-2} with 1 shot. The size of the hole was bigger than that of the original mask (30 μm × 30 μm). The etching depth was 950 nm. The periodic structure, which was observed on the irradiated surface of the DF, was not observed on the irradiated surface of the HF. This result suggested that the laser ablation of the HF did not mainly originate from the laser intensity profile of the irradiated region. Fig. 2 shows plots of the etching rate (etch depth/pulse) versus fluence for the DF and HF. The linear relationships were in good agreement with the well-known relationship between the etching rate and the fluence based on Lambert–Beer's law. The etching rate for the HF was about five times greater than that for the DF. This would result from the difference in the absorption coefficients between the DF and HF. The threshold fluences were ca. 30 mJ cm^{-2} for the DF and ca. 20 mJ cm^{-2} for the HF. The HF has a smaller threshold fluence than the DF. In general, the ablation threshold increased with the decreasing absorption coefficient for the doped polymer or different wavelength. However, in spite of the decrease in the absorption coefficient for HF by swelling, the ablation threshold decreased in comparison with the DF. Therefore, this behavior suggested that the ablation mechanism changed for the HF duo to the water

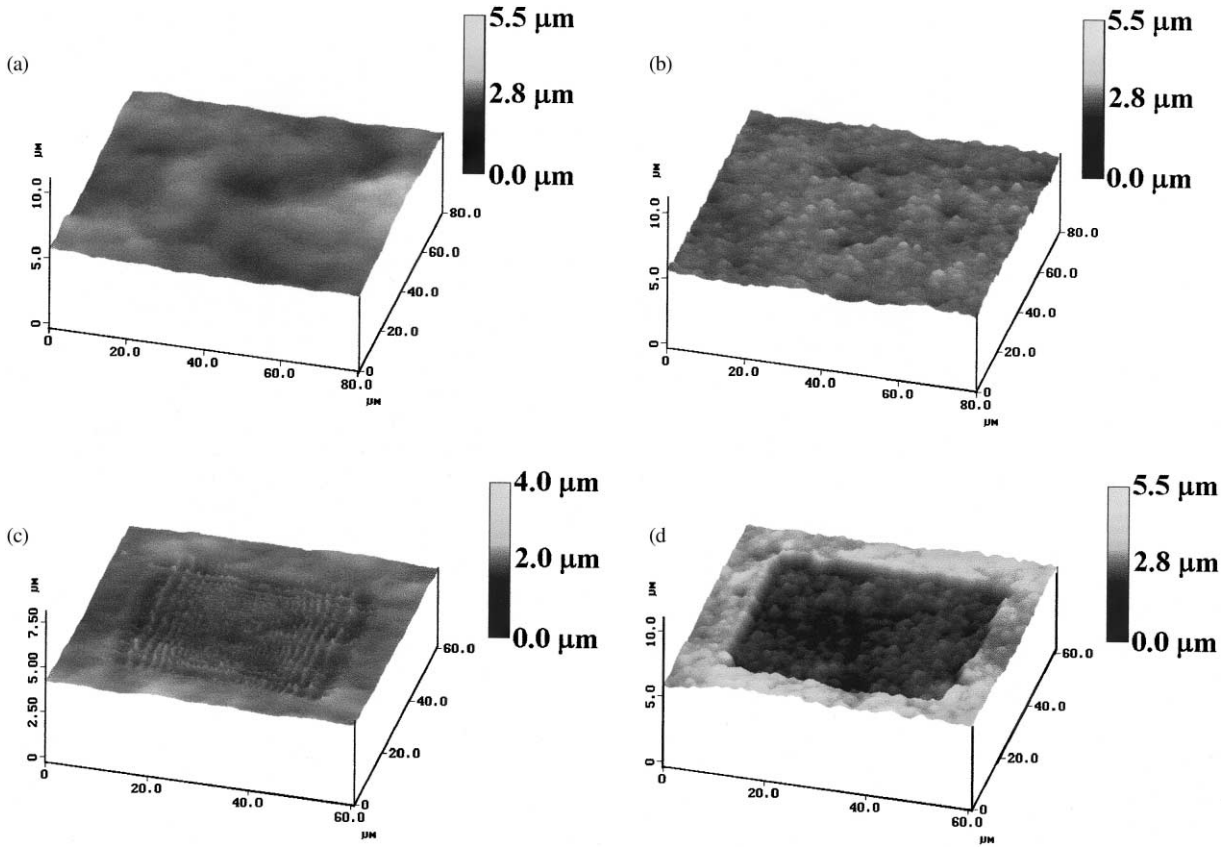

Fig. 1. AFM images of the surface of non-irradiated (a) DF and (b) HF, and AFM images of (c) DF and (d) HF irradiated at 0.10 J cm^{-2} with 1 pulse.

in the gel matrix. The laser irradiation below the threshold of the DF could not generate sufficient bond scission for ablation, however, the local heat and pressure generated by the laser irradiation was sufficient for bubble formation.

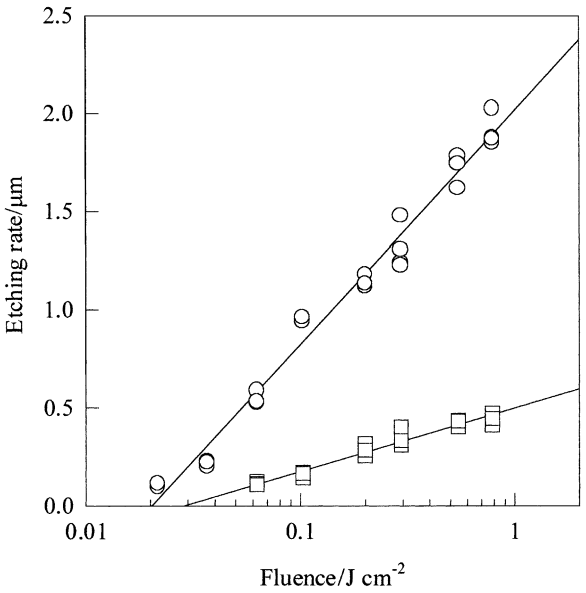

Fig. 2. Etching rates of DF (□) and HF (○) as a function of laser fluence.

Several researchers observed bubble formation in the region of the hydrated gel and tissue irradiated with the excimer laser and IR-laser [8,11,12]. They suggested that the ablation is brought about by the collapse of a bubble induced by the boiled water or cavitation. It was predicted that the effect of bubble formation for the HF contributes to the etching and inferred that the irregularity of the surface structure for the HF (see Fig. 1(d)) may be created by the collapse of bubbles generated by cavitation or the boiling of water in the gel matrix.

3.3. Time-resolved photographic study

Fig. 3 shows time-resolved photographs of the laser irradiation for the DF at a fluence of 0.20 J cm^{-2}. The shock wave was observed from 50 ns to 1 μs on the ablation site. However, ejected materials were not observed in this time period. The ejected materials would be too small due to their decomposition to be detected by this method. Fig. 4 shows time-resolved photographs of the laser irradiation for the HF at a fluence of 0.20 J cm^{-2}. As for the DF, the shock wave was observed over the period from 50 ns to 1 μs on the ablation site. Moreover, a significant amount of ejected material was observed in the photographs. The ejected materials were detected in the period of 300 ns to 100 μs on the ablation site. The shape of the ejected materials was like a

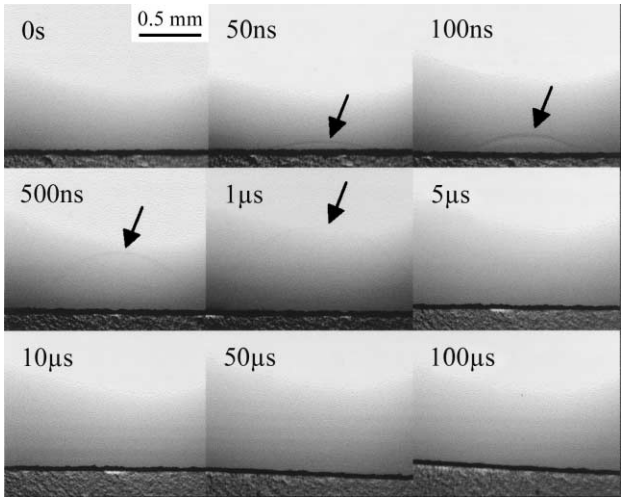

Fig. 3. Time-resolved photographs of the DF at different time delays after the laser irradiation at 0.20 J cm^{-2}. The arrows indicate the shock wave front.

"mushroom cloud" at 10 μs. The shape of the ejected materials changed with the fluence. These detailed results will be reported elsewhere. The main component of the ejected materials was water, because the HF contained about 90 wt.% water. The presence of water in the ejected materials was recognized by the color change of the water-coordinated cobalt complex. This concept was supported by light scattering measurements. Hahn et al. [13] have reported that the total volume of water in the ejected materials was in good agreement with the water content in the ablated corneal tissue. The presence of water in the HF would change the decomposition mechanism and give rise to the ejecting of measurable-sized particulates.

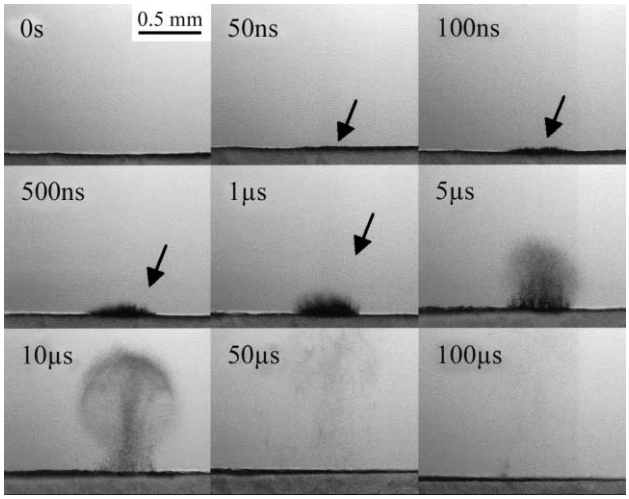

Fig. 4. Time-resolved photographs of the HF at different time delays after the laser irradiation at 0.20 J cm^{-2}. The arrows indicate the shock wave front.

3.4. Evaluation for temperature rise

In order to confirm the temperature rise, the temperature of the ablated surface was calculated. The temperature rise, $T(x)$, of the HF surface could be roughly estimated, based on the assumption that the absorbed laser energy was converted entirely into heat and that the heat diffusion was much slower than the laser pulse duration [14]:

$$T(x) = T_r + \frac{\alpha_{eff} F}{\rho C} e^{-\alpha_{eff} x} \quad (1)$$

where x is the position relative to the initial surface position, α_{eff} the effective absorption coefficient, F the incident laser fluence, T_r the preirradiation sample temperature, and ρ and C the density and the heat capacity, respectively. The values of ρ and C of an HF at room temperature were 1.0 g cm^{-3} and 3.9 J g^{-1} K^{-1} [15], respectively. α_{eff} of the HF was estimated to be 1.9×10^4 cm^{-1} from Fig. 2. $T(x)$ is the maximum possible temperature if the water in the HF was superheated without evaporation. The calculated $T(x)$ for the HF at $F = 0.10$ J cm^{-2} and $x = 950$ nm, which is the etching depth at 0.10 J cm^{-2}, was ca. 100°C. This temperature is significantly higher than the denaturation temperature of collagen gel, however, the temperature is below the boiling point of water in a two-component system. Therefore, the cavitation of water would play a very important role in the laser ablation of the HF.

3.5. FTIR–ATR spectroscopy

In order to investigate the thermal effects on the microscopic structures of the collagen molecules on the ablation site, the degree of denaturation was scrutinized using FTIR–ATR spectroscopy. Fig. 5(a) and (b) shows the FTIR–ATR spectra for the collagen and gelatin films. These spectra agree with the spectra in a previous report [16]. Fig. 6(a) and (b) shows the FTIR–ATR spectra of the amide I bands of the DF and HF, respectively, after laser irradiation at a fluence of 0.10 J cm^{-2} with 1, 2, 3, and 5 shots. For comparison, the FTIR–ATR spectra of the amide I band of the collagen and gelatin are shown in both Fig. 6(a) and (b). The peak shifted to a lower wavenumber with the laser shots for the DF. This result indicated that the laser irradiation caused denaturation of the collagen on the ablated site and the degree of denaturation increased with the increasing number of laser shots. However, this measurement could be done up to 5 laser shots and then the IR spectra was not measurable. This is because a large surface roughness prevented the FTIR–ATR spectral measurements. On the other hand, the peak shift for the HF was smaller than that for the DF. Fig. 7(a) and (b) shows the Fourier self-deconvoluted spectra of the amide I band of the collagen and gelatin, respectively. Six peaks were well resolved for the collagen and gelatin by resolution enhancement. Each of the component bands of collagen resolved by the curve fitting is assigned to the vibrational mode associated with different secondary

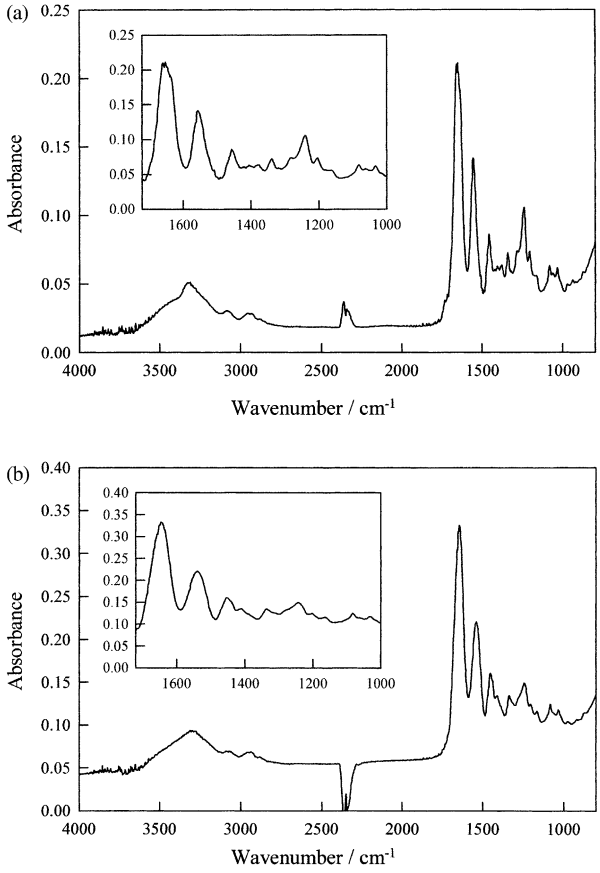

Fig. 5. IR spectra of (a) collagen and (b) gelatin films.

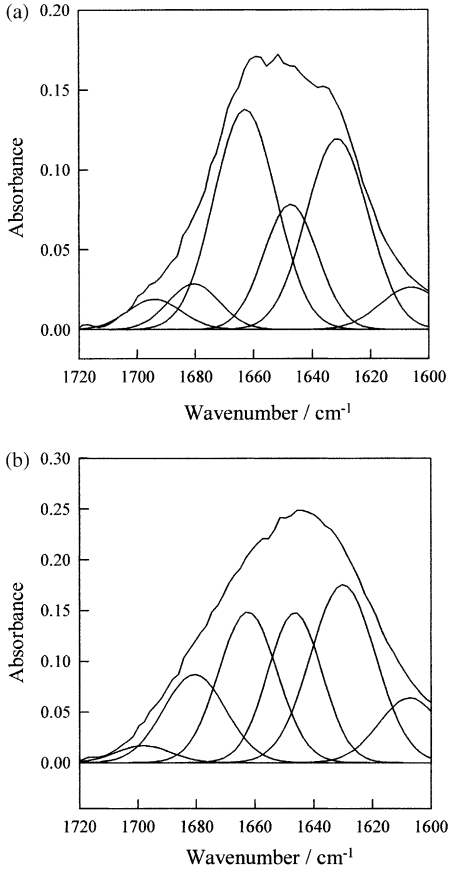

Fig. 7. Components of the amide I absorption band of (a) collagen and (b) gelatin.

structures. The intensity of the peak at 1661 cm^{-1} assigned to a helix-related hydrogen-bonded set of carbonyls [16] was stronger than that at 1633 cm^{-1} assigned to disorder [17], because the collagen molecule has a hydrogen-bonded triple-helix structure. As for the gelatin, the assignment of the peaks was the same as that for the collagen. However, unlike collagen, the intensity of the peak at 1661 cm^{-1} was weaker than that at 1633 cm^{-1}. This result indicated that the denaturation decreased the C=O group, which constitutes the peak of the hydrogen-bonded triple-helix structures, and increased the C=O group which is part of the disordered structure. The ratio of the area of the peak at 1661 cm^{-1} to

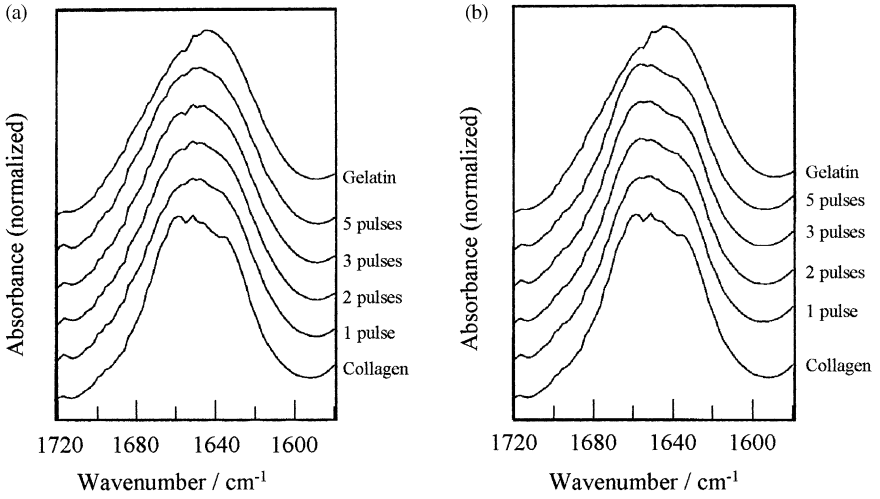

Fig. 6. Amide I absorption band of the IR spectra of (a) DF and (b) HF irradiated at 0.10 J cm^{-2}.

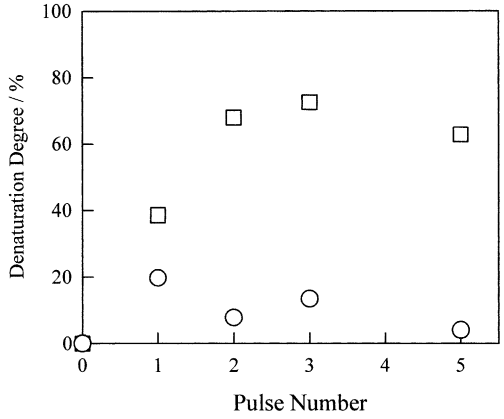

Fig. 8. Plot of denaturation degree as a function of pulse number for the DF (□) and HF (○) irradiated at 0.10 J cm^{-2}.

that at 1633 cm^{-1} (A_{1661}/A_{1633}) was calculated for assessment of the degree of denaturation on the laser irradiated surface. Fig. 8 shows the A_{1661}/A_{1633} ratio of the DF and HF after laser irradiation at a fluence of 0.10 J cm^{-2} with 1, 2, 3, and 5 shots. Here, we defined the ratio for collagen as 0 and that for gelatin as 100. The A_{1661}/A_{1633} ratio of the DF increased with the increasing number of laser shots. The degree of denaturation was 40% with 1 shot, and then increased to 70% after 5 shots. On the other hand, the A_{1661}/A_{1633} ratio of the HF slightly increased with the increasing number of laser shots. The degree of denaturation was 10% even after 5 laser shots. The denaturation temperature of the collagen drops to 48°C from 200°C by swelling. This drop in the denaturation temperature suggested that the degree of denaturation increased more in the HF than in the DF at the same fluence. However, from the FTIR–ATR measurement, the degree of denaturation for the HF was smaller than that for the DF at the same fluence. Thus, part of denatured collagen would be evaporated by the collapse of the bubble, or the water in the HF would suppress the temperature rise of the laser irradiation part of the HF due to the high enthalpy of the vaporization of water.

4. Conclusion

The water contribution to excimer laser ablation of collagen was investigated by time-resolved photography and ATR–FTIR. During the ablation process for HF, the laser energy would be mostly consumed as the latent heat of evaporation of water, that is, the water in the gel matrix would contribute to the suppression of the increment in the temperature in the irradiated region. The collapse of a bubble induced by the boiled water or cavitation would be important part of the ablation of HF.

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